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MUKRIMIN MUKRIMIN

HETEROBASIDION — CONIFER PATHOSYSTEM: HOST MYCOBIOME AND EVALUATION OF POTENTIAL RESISTANCE MARKERS

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Heterobasidion – conifer pathosystem: Host mycobiome and evaluation of potential resistance markers

Mukrimin

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Cover: An asymptomatic tree of Norway spruce (without visible decay symptoms) (left). A symptomatic tree (with well-defined decay symptoms) (right)

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ABBREVIATIONS

ABA	abscisic acid
cDNA	complementary deoxyribonucleic acid
DAMPs	damage-associated molecular patterns
DNA	deoxyribonucleic acid
DW	dry weight
ET	ethylene
ETI	effector-triggered immunity
FT-IR	Fourier-Transform Infrared
GPPS	geranyl diphosphate synthase
GWAS	genome-wide association study
JA	jasmonic acid
MC5	metacaspase 5
OTUs	operational taxonomic units
PAMPs	pathogen-associated molecular patterns
PCD	programmed cell death
PCoA	principal coordinates analysis
PCR	polymerase chain reaction
PLSR	partial least squares regression
PRRs	pattern recognition receptors
PTI	PAMPs-triggered immunity
q-PCR	real-time quantitative PCR
R gene	resistance gene
RNA	ribonucleic acid
SA	salicylic acid
SAR	systemic acquired resistance
SIMCA	soft independent modeling of class analogy
SNPs	single nucleotide polymorphisms

LIST OF ORIGINAL PUBLICATIONS

This present thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV):

I. A. Kovalchuk*, **M. Mukrimin***, Z. Zeng, T. Raffaello, M. Liu, R. Kasanen, H. Sun, F.O. Asiegbu. Mycobiome analysis of asymptomatic and symptomatic Norway spruce trees naturally infected by the conifer pathogens *Heterobasidion* spp. Environmental Microbiology Reports (2018), 10(5):532–541. https://onlinelibrary.wiley.com/doi/full/10.1111/1758-2229.12654. (*Co-first authors).

II. **M. Mukrimin**, A. Kovalchuk, L.G. Neves, E. Jaber, M. Haapanen, M. Kirst, F.O. Asiegbu. Genome-wide exon-capture approach identifies genetic variants of Norway spruce genes associated with susceptibility to *Heterobasidion parviporum* infection. Frontiers in Plant Science (2018), 9:358. https://doi.org/10.3389/fpls.2018.00793.

III. **M. Mukrimin**, A. Kovalchuk, R.P. Ghimire, M. Kivimäenpää, H. Sun, J. Holopainen, and F.O. Asiegbu. Evaluation of potential genetic and chemical markers for Scots pine tolerance against *Heterobasidion annosum* infection. Planta (2019). https://link.springer.com/article/10.1007%2Fs00425-019-03270-8.

IV. **M. Mukrimin**, A.O. Conrad, A. Kovalchuk, R. Julkunen-Tiitto, P. Bonello, and F.O. Asiegbu. Fourier-Transform Infrared (FT-IR) spectroscopy analysis discriminates asymptomatic and symptomatic Norway spruce trees. Plant Science (2019), 289:110247. https://doi.org/10.1016/j.plantsci.2019.110247.

OTHER ARTICLES NOT INCLUDED IN THIS THESIS

- F. Ren, A. Kovalchuk, **M. Mukrimin**, M. Liu, Z. Zeng, R.P. Ghimire, M. Kivimäenpää, J.K. Holopainen, H. Sun, F.O. Asiegbu. Tissue microbiome of Norway spruce affected by *Heterobasidion*-induced wood decay. Microbial Ecology (2018), https://doi.org/10.1007/s00248-018-1240-y.

- A. Kovalchuk, Z. Zeng, R.P. Ghimire, M. Kivimäenpää, T. Raffaello, M. Liu, **M. Mukrimin**, R. Kasanen, H. Sun, R. Julkunen-tiitto, J.K. Holopainen, F.O. Asiegbu. Dual RNA-seq analysis provides new insights into interactions between Norway spruce and necrotrophic pathogen *Heterobasidion annosum s.l.* BMC Plant Biology (2019), 19:2, https://doi.org/10.1186/s12870-018-1602-0.

AUTHOR CONTRIBUTIONS

I. **MM** performed the fieldwork and laboratory work (DNA extraction), contributed to the data analysis, and prepared manuscript draft together with other authors.

II. **MM** performed the greenhouse work (phenotyping of Norway spruce saplings) and laboratory work (DNA extraction) and contributed to the data analysis and to the preparation of manuscript draft together with other authors.

III. **MM** performed the fieldwork and laboratory work and contributed to the data analysis and wrote the manuscript together with other authors.

IV. **MM** performed the fieldwork and laboratory work, contributed to the data analysis, and wrote the manuscript together with other authors.

ABSTRACT

Forests are the hotspots of Earth's biodiversity, providing habitats to animals and resources to humans, protecting watersheds, preventing soil degradation, and mitigating climate change. In fact, forest disturbance is mainly caused by biotic and abiotic stresses, which affect both the primary metabolic components required for growth, development, and reproduction and secondary metabolites (defense-related chemical compounds) of trees. In the Northern Hemisphere, including Finland, members of the fungal group *Heterobasidion annosum* species complex are the most important pathogens of conifer trees causing serious economic losses for forest industries. The existing control and management strategies against this pathogen do not lead to 100% protection.

To gain better insights and knowledge of *Heterobasidion*–conifer tree interactions, I investigated the impact of pathogen infection on the resident mycobiota in naturally infected trees as well as performed an analysis of fungal community structure under field conditions. Fourier transform infrared spectroscopy (FT-IR) analysis was also performed to discriminate between asymptomatic and symptomatic trees. In a parallel study, I investigated the genetic variants that could be associated with the control of necrotic lesions caused by *H. parviporum* inoculation among selected clonal lines of Norway spruce. Additionally, the expression level of a subset of selected genes involved in terpene, stilbene and flavonoid biosynthesis and programmed cell death in Scots pine trees with varying levels of resistance was further assessed.

Mycobiome analysis demonstrated significant differences in the structure of fungal communities residing within symptomatic and asymptomatic Norway spruce trees. The results provided novel insight into the interactions between fungal plant pathogens and resident plant mycobiota. The FT-IR spectroscopy analysis was able to discriminate between

symptomatic and asymptomatic *Heterobasidion*-infected Norway spruce trees. Other findings in terms of genetic and chemical markers revealed ten single nucleotide polymorphisms (SNPs) associated with eight genes. The identified SNPs were significantly associated with larger lesions in response to *H. parviporum* inoculation in Norway spruce saplings. In Scots pine, genes with higher expression levels predicted to encode α-pinene synthase, geranyl diphosphate synthase (GPPS), and metacaspase 5 (MC5) were associated with trees exhibiting high levels of necrotic lesion formation in response to fungal inoculation. Concentrations of two terpenoid compounds (β-caryophyllene and α-humulene) were significantly negatively correlated with lesion size. These results can be used in further studies to elucidate potential biomarkers in conifer tree genetic resistance research.

Keywords: *Heterobasidion*, Norway spruce, Scots pine, tree-pathogen interactions, mycobiome analysis, FT-IR spectroscopy, gene expression, terpenoid, chemical markers

1. INTRODUCTION

1.1. Forest as a natural resource

Forests, covering approximately 30% of the world's land area and accumulating 80% of the total plant biomass, are the hosts of global biodiversity (https://www.un.org/sustainabledevelopment/biodiversity/). Forests are classified/subdivided into boreal, temperate, Mediterranean, subtropical, and tropical forests. Boreal forest, also known as taiga, is the largest land biome on our planet. It is located on a wide range of continents, e.g., in North America (inland Canada, Alaska, and the northern contiguous United States of America) and in Eurasia (from the Atlantic coast of Norway to the Russian Pacific coast) (Metla, 2005). Boreal forest is mostly dominated by conifers and several broad-leaved species. In Finland alone, boreal forest comprises 67% Scots pine (*Pinus sylvestris*), 22% Norway spruce (*Picea abies*), and 11% broad-leaved trees, including birches (*Betula* spp.) (Metla, 2013; http://www.metla.fi/metinfo/sustainability/c4-tree-species.htm).

Forests provide habitats for animals and resources for societies as well as support humans' livelihoods and leisure. Forests also protect watersheds, hold water, prevent soil erosion, mitigate climate change, and perform many other ecological functions. In addition, forests' global economic value is estimated to be more than 600 billion dollars, and they employ over 13 million people (Li et al., 2019). The annual average production of forest products, such as timber, is estimated to be 3 billion cubic meters (FAO, 2016).

Furthermore, through photosynthesis, forests can produce O_2 and absorb CO_2; therefore, forest disturbance might affect tree biochemical and physiological processes. It can trigger higher CO_2 concentrations in the atmosphere and exacerbate climate change. Consequently, such disturbances affect plant growth and responses to biotic and abiotic stresses. The biotic stresses in forests may be caused by fungal pathogens, viruses, bacteria,

insects, mites, and herbivores among other organisms, whereas the abiotic stresses may be caused by temperature, drought/flooding, marginal and poor soils, toxic chemicals, wind, storms, snow, nutrient imbalance, and air pollutants (e.g., SO_2), among other factors.

1.2. Forest tree-pathogen interactions

Organisms in forest ecosystems, including fungi, are involved in numerous inter- and intraspecific interactions. For instance, the life of pathogenic fungi is highly dependent on other organisms, e.g., plant hosts that these fungi use as a food resource. Plant and organic debris are the main nutrient and carbon source for the growth and development of fungi. Plant-fungi interactions involve organisms with varying lifestyle modes, such as mutualists, parasites, and commensals (Hirsch, 2004). Plant-fungi mutualism is a form of interaction in which the plant and fungi exchange nutrients (e.g., mycorrhiza) and/or live together to the mutual benefit of both partners. In the case of parasitism, parasitic fungi obtain nutrients from another organism (living cells) and harm the host in some manner. Commensalism refers to a kind of association between two organisms in which one partner benefits and the other derives neither harm nor benefit (Terhonen et al., 2019; Volk, 2013). Another life mode involves saprobes, which are fungi using dead organic materials as their food sources (Saikkonen, 2007; Volk, 2013).

Parasitic fungi can be further subdivided into biotrophs, necrotrophs, and hemibiotrophs. Biotrophic fungi feed on living host tissue for their growth and reproduction, causing symptoms of disease in certain host tissues. Necrotrophs kill host cells before feeding on the dead host tissues (Geeta and Mishra, 2018; Spanu and Panstruga, 2017). Hemibiotrophs act either as biotrophs or as necrotrophs depending on their life cycle stage and the conditions: typically, they initially feed on living tissues for some time and then on dead tissue (Surico, 2013).

Interactions between trees (hosts) and pathogens (fungi) over evolutionary time have been studied by Vacher et al. (2008). The study indicated that the deep evolutionary history of fungal species can be described by the very early divergence of Ascomycota and Basidiomycota (Vacher et al., 2008). The Ascomycota and Basidiomycota recently emerged as parasites of angiosperms (Magnoliophyta) and gymnosperms (Coniferophyta) in the last 140-180 million years ago (mya) (Dalman, 2010). The oldest known fossil records of ascomycetous fungi are from 400 mya, whereas the divergence of these two fungal phyla was estimated to have occurred 722 mya (Dalman et al., 2010). Furthermore, these two phyla have colonized both angiosperms and gymnosperms, and these interactions may represent the evolutionary origin of tree-fungi interactions.

1.3. The tree immune system

The tree immune system refers to the ability of trees to identify and defend themselves against numerous pathogens (fungi and insects) (Coll et al., 2011; Grandellis et al., 2019). In their coevolutionary history, trees have successfully developed several defense strategies to resist pathogenic attack. Despite these active defenses, pathogens are still able to infiltrate the surface of tree tissues by secreting hormones, enzymes, and toxic proteins (Kovalchuk et al., 2013; Raffaello and Asiegbu, 2017).

The first layer of the tree defense system includes physical barriers. After pathogens penetrate the physical barriers, they must confront the second defense layer, namely, the tree immune system, called innate immunity (Grandellis et al., 2019). The innate immune system of plants can act to distinguish self from non-self as well as activate strongly regulated defense responses to reduce damage caused by the invaders (Coll et al., 2011).

The ‘zig-zag’ model proposed by earlier authors (Jones and Dangl, 2006) is a classical model of the interaction between invading pathogens and the plant immune system. The zig-

zag model postulates that there are two innate immunity lines against the attacks of pathogens consisting of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI). Transmembrane pattern recognition receptors (PRRs) recognize PAMPs and trigger PTI, while ETI is directly or indirectly mediated by resistance genes (R genes), which is an interaction between pathogen effectors and plant receptor proteins. Both ETI and PTI are mediated by signaling pathways: ethylene (ET), salicylic acid (SA), and jasmonic acid (JA) (Han, 2019). In the biotrophic pathosystem, programmed cell death (PCD) and systemic acquired resistance (SAR) are triggered by R genes in plants. JA and ET are involved in defense mechanisms against necrotrophic pathogens, whereas biotrophic pathogens lead to SA pathway induction (Spoel et al., 2007). In addition, another trigger used to detect potential pathogen invasion involves damage-associated molecular patterns (DAMPs), which are specific proteins released from dead or damaged plant cells (Rubartelli and Lotze, 2007).

1.4. Resistance and defense response of trees

1.4.1. Defence mechanisms

A tree has a remarkable ability to handle or manage both biotic and abiotic stresses. Resistance and tolerance are two terms commonly used to define how trees manage these stresses. Resistance is a heritable trait of trees that can restrict the effect of potential pests and pathogens, whereas tolerance refers to the ability of trees to grow normally and productively, despite being under attack by insects and pathogens (Katjiua and Ward, 2006).

Plants, including trees, are targets of attack by a wide range of pathogens and herbivores. Trees have developed various defense mechanisms comprising physical and chemical barriers that defend against arthropod pests and pathogens. The mechanical or physical barriers (physical defenses) are the first defense layer of trees. To protect themselves

from their enemies, trees often have spines, thorns, hairs, waxes, cutins, suberins, cuticular lipids, and lignified cells that can prevent/impede invasion by pathogens (Castells, 2015).

Furthermore, trees also have outer bark that protects the cambium and phloem tissues (inner bark) (Kovalchuk et al., 2013). In fact, the trees' mechanical barriers can be penetrated by only a few invaders through wounded or damaged tree tissues, e.g., surface wounds and the freshly exposed wood surfaces of stumps. The defensive secondary metabolites produced by trees include phenolics (e.g., lignin, condensed tannins, phenolic glycosides, and bound phenolics), terpenes, defense-related proteins (e.g.., chitinases, defensins, polyphenol oxidase, and peroxidases), and N-containing compounds (e.g., alkaloids) (Bhatia and Vishwavidyalya, 2015; Wink, 2015).

1.4.2. Terpenes and phenolics as secondary metabolites

Secondary metabolites often are not considered essential components of plant metabolism as they usually are not critically involved in growth, photosynthesis, respiration, development, and reproduction (roles of primary metabolites). Secondary metabolites are, however, very important for survival purposes. Secondary metabolites constitute a part of the defense response in both the first barrier (perimeter protection) and the second barrier (chemical defense) of trees that can inhibit herbivores, reduce tissue loss, and attract pollinators and predators that feed on herbivores.

The three major groups of secondary metabolites are terpenes, alkaloids, and phenolic compounds. In conifers, the majority of these compounds are produced and deposited in resin canals and polyphenolic parenchyma cells (Chomel et al., 2016). Terpenes are the most important plant defensive chemicals (both chemical and physical defensive compounds) against invading and attacking organisms, such as pathogenic fungi and bacteria, insects, and herbivores (Peter, 2018). Terpenes are also constituents of essential oils.

Based on the number of isoprene units, terpenes are divided into three groups: mono-, sesqui- and diterpenes, with 10, 15, and 20 carbon atoms, respectively. Several monoterpenes are found in trees, including conifers, but α-pinene, β-pinene, and δ-3-carene are the main volatile organic compounds (VOCs) due to their higher abundance. Furthermore, these monoterpenes have been investigated and found to be directly regulated by host-tree genetics (Romón et al., 2017).

In addition, trees release VOCs through their leaves or needles, which serve critical functions in growth, survival, and defense (Geeta and Mishra, 2018). A large proportion of these VOCs are terpenes, either monoterpenes or sesquiterpenes, and these compounds are highly diverse both within and among plant species (Courtois et al., 2012). Trees also produce non-VOCs called diterpenes.

Most conifers can produce oleoresin, which is a viscous and odoriferous liquid containing approximately equal amounts of mono- and diterpenes and smaller amounts of sesquiterpene compounds (Keeling and Bohlmann, 2006). Terpenoid oleoresin plays a role in conifer defense as it is mobilized to wounded tree tissues, which acts to heal the wound. Oleoresin stored in resin canals can trap and prevent insects from entering the site.

Phenolic metabolites in forest trees play a key role in defense against abiotic and biotic stressors, including herbivores and pathogens. Phenolics induced by insect and pathogen attacks increase the probability of host survival (Rigsby et al., 2019). Furthermore, each tree tissue varies in the composition of phenolic compounds, e.g., between stems and needles (leaves) of tree species, such as birch (*Betula alba*) and Norway spruce (*Picea abies*) in boreal forest (Ghimire et al., 2018). Furthermore, the seasonal variation of phenolic metabolites is much greater in conifers, e.g., in needles of Scots pine (*Pinus sylvestris* L.) (Nerg et al., 1994).

Phytoalexins, derived phenols that accumulate in trees under numerous stress factors, are toxic to pathogens. Phytoalexins can stop the growth of pathogens and provoke defense reactions in living cells. Another type of derived phenol, chlorogenic acids, are strong fungicides, bactericides, and virucides with a direct antibiotic effect that further facilitates the tree resistance to pathogens (Nawrot, 2013).

1.5. *Heterobasidion annosum* species complex

1.5.1. Taxonomy of *H. annosum*

Currently, the *H. annosum* species complex is classified into five aggressive pathogens that differ in morphology, ecology, intersterility, and molecular characters: *H. annosum s.s.*, *H. parviporum*, *H. abietinum*, *H. occidentale*, and *H. irregulare*. These species cause root and butt rot, mainly in conifers in the Northern Hemisphere, including the boreal forest zone (Rodriguez et al., 2013; Worrall et al., 2010). The European group comprises three species of *Heterobasidion*: *H. annosum*, *H. parviporum*, and *H. abietinum*, of which the main hosts are Scots pine, Norway spruce and silver fir (*Abies alba*), respectively, whereas the North American species are *H. irregulare* and *H. occidentale* (Abu et al., 2004; Asiegbu et al., 2005; Garbelotto and Gonthier, 2013) (**Fig. 1**).

From a historical perspective, the most recent ancestor of the genus *Heterobasidion* is estimated to have appeared approximately 160 mya, which is consistent with the presence of gymnosperms. The ancestor diverged approximately 75–85 mya into two groups: *H. annosum s.s./H. irregulare* and *H. parviporum/H. abietinum/H. occidentale*. The first group includes pine-occupying fungi, and this evolutionary divergence is in line with the evolutionary divergence of the host genera *Pinus* and *Picea*, whereas the second group includes non-pine-infecting fungi. However, this estimated evolutionary separation does not correlate with the divergence between *Pinus* and *Picea* (Lind et al., 2014b).

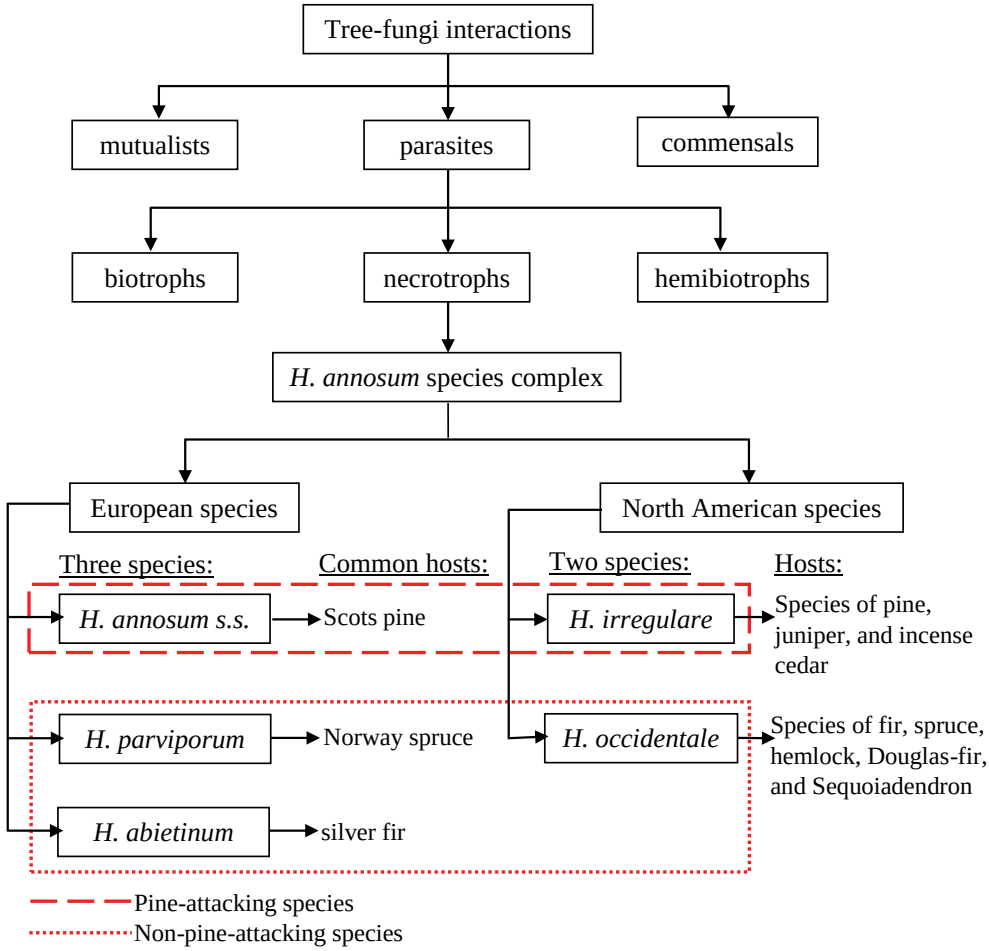

Fig. 1. Illustration of tree-fungi interactions between the *Heterobasidion annosum* species complex and tree hosts, showing that the *H. annosum* species complex includes three species in Europe and two species in North America with two main hosts (pine and non-pine trees)

The ancestral split of the group *H. annosum s.s./H. irregulare* into different species occurred approximately 30 mya. This divergence is probably attributable to the disappearance of the land bridge between North America and Europe, which is predicted to have occurred approximately 35-50 mya. The evolution of the most recent common ancestor of the *H. occidentale/H. parviporum/H. abietinum* group occurred approximately 30–40 mya. In addition, the current distribution of *H. parviporum* in Europe might be the result of

recolonization with its host at the end of glaciation. *H. parviporum* is more closely related to *H. abietinum* than to other members of the species complex. *H. abietinum* emerged approximately 20 million years ago, with *Abies* species as the main host. In contrast, it is estimated that *H. annosum* s.s. began to split from *H. irregulare* approximately 30 million years ago (Dalman, 2010; Dalman et al., 2010).

The first published report of *H. annosum* as a causative agent of root and butt rot disease of conifers was by Hartig in 1874 as reported by Garbelotto and Gonthier (2013). In the past several decades, studies have been completed on the phylogenetic relationships of the *H. annosum* species complex based on different genetic markers (e.g., Dalman, 2010; Dalman et al., 2010). Research in several European countries, e.g., Fennoscandia, Belarus, Britain, Bulgaria, Estonia, Italy, Lithuania, Poland, Slovenia, Spain, and Switzerland, has also resulted in at least 1,700 scientific papers on this pathogen. Furthermore, *H. annosum* s.l. was the first tree-pathogenic homobasidiomycete with a complete genome sequence (Olson et al., 2012). The size of the sequenced *H. irregulare* genome is 33.6 million base pairs (Mbp); it contains 13,405 predicted genes distributed on 14 chromosomes (https://genome.jgi.doe.gov/Hetan2/Hetan2.info.html). The genome of *H. parviporum* has recently been published (Zeng et al., 2018).

The economic damage caused by the *H. annosum* species complex, particularly in the Northern Hemisphere, including the boreal forest zone, is substantial. The annual economic losses for the Finnish and European forest industries are estimated to be €50 million and €790 million, respectively (Asiegbu et al 2005; Gori et al., 2013; Lind et al., 2014a; Piri and Hamberg, 2015). In Finnish forest areas, *H. annosum* s.s. and *H. parviporum* impact the growth and wood properties of Scots pine and Norway spruce and their further use for various industrial purposes (Rodriguez et al., 2013).

1.5.2. Infection biology of *Heterobasidion annosum s.l.*

The process of *H. annosum s.l.* infection comprises two mechanisms: primary and secondary infections. The primary infection is started by wind-carried basidiospores of *H. annosum* landing on either freshly cut stump surfaces or wounds on trees, followed by germination, mating, and the formation of heterokaryotic mycelium (Asiegbu et al., 2005). Secondary infections occur when the formed mycelium grows into the root system via the stump and then spreads to neighboring healthy trees by root-to-root contact (Sedlák and Tomšovský, 2014). In addition, basidiospores can typically disperse up to hundreds of meters (0.1 to 1.25 km) away by wind/air flows, until they land on freshly cut stump surfaces and fresh wounds of trees (Garbelotto and Gonthier, 2013). *Heterobasidion* also produces conidiospores (asexual spore) during its life cycle which can be vectored by insects or easily transferred for short distances to new substrates (Asiegbu et al., 2005). Under field conditions, both basidiospores and conidiospores are produced, but the precise role of asexual spores in stump infection in nature is yet not fully clarified.

1.5.3. Control and management strategies of *Heterobasidion annosum s.l.* infection

H. annosum s.l. is a tree pathogen that can be controlled in managed forests (Garbelotto and Gonthier, 2013) by mechanical, chemical, and biological approaches. Currently, the pathogen is controlled by applying silvicultural treatments, biological control agents (e.g., *Phlebiopsis gigantea*), and chemicals (e.g., urea and borates). Regrettably, these treatments do not lead to 100% protection (Möykkynen et al., 1998). However, those control methods can decrease the spread and transfer of mycelial growth and minimize economic losses.

Silvicultural or mechanical strategies can be applied by removing stumps and roots to limit airborne and root-to-root spread of infections. Other measures include the use of broad-

leaved trees to replace susceptible tree conifers since broad-leaved species have relatively low susceptibility to the pathogens. Additionally, performing thinning and logging operations during winter can help lower the rate of spore infection (Garbelotto and Gonthier, 2013).

Biological control can be carried out using the fungal species *P. gigantea*, which is an antagonist or a competitor of *H. annosum s.l.*, leading to satisfactory reduction of pathogen infection. A *P. gigantea* spore suspension can be applied to fresh stumps immediately after tree felling. Presently, this is an effective and environmentally friendly alternative method used to control and manage *Heterobasidion* root and butt rot (Sun et al., 2013; Terhonen et al., 2013). Chemical control involves applying urea and borates immediately to fresh stump surfaces during logging operations (Berglund, 2005). It is very efficient, but chemical control is associated with environmental concerns since the chemical agents might negatively affect the surrounding vegetation and other organisms. In the long term, resistance based on genetics should offer more durable and reliable control.

2. AIMS OF THE STUDY

The objectives of this study were to

a. Investigate the composition of the fungal communities associated with different anatomic regions of Norway spruce (I);
b. Assess the impact of root and butt rot caused by *Heterobasidion sp.* on the structure of these communities under field conditions (I);
c. Evaluate genetic polymorphism among selected clonal lines of Norway spruce (II);
d. Identify genetic variants associated with the control of necrotic lesion lengths caused by *H. parviporum* inoculation (II);
e. Determine the responses of naturally regenerated mature Scots pine trees to *H. annosum* inoculation under field conditions (III);
f. Assess the expression level of selected genes involved in terpene, stilbene and flavonoid biosynthesis and programmed cell death in Scots pine trees with varying levels of resistance (III); and
g. Evaluate the applicability of Fourier-transform infrared (FT-IR) spectroscopy for the identification of symptomatic and asymptomatic Norway spruce trees (IV).

The hypotheses of this study were as follows:

a. Colonization of Norway spruce trees by *Heterobasidion* fungi affects the structure of resident fungal communities (I)
b. An exon sequence-capture approach can be used to identify genetic loci of Norway spruce associated with susceptibility or resistance to *H. parviporum* infection (II)
c. Terpenoid compounds play a role in the resistance of Scots pine trees to *H. annosum* infection (III)
d. FT-IR spectroscopy can be used as a tool to distinguish between asymptomatic and symptomatic Norway spruce trees (IV)

3. MATERIALS AND METHODS

The tree species, fungal species and strains, and associated methods used are summarized in Tables 1 and 2. Detailed explanations can be found in both published articles and submitted manuscripts attached to this thesis.

3.1. Materials

Table 1: Tree species used in this study

Tree species	Information	Publications
Norway spruce (*Picea abies* (L.) H. Karst.)[a]	Mature trees (*ca* 55 years old), needles (*ca* second year), upper stem, lower stem, bark, phloem, and xylem	I, IV
Norway spruce[b]	Clonal saplings (3 years old), phloem, xylem, and needles (*ca* one-year-old)	II
Scots pine (*Pinus sylvestris* L.)[c]	Mature trees (10-15 years old), needles (*ca* second year), and stem	III

[a] Study site: privately owned managed forest in the municipality of Mäntsälä, Uusimaa region, southern Finland (60°44′51″N, 25°13′17″E; 60°45′11″N, 25°13′24″E; and 60°45′15″ N, 25°15′34″ E)

[b] Study site: progeny trials in Pieksämäki (N 62°23′, E 27°17′)

[c] Study site: Research forest site in Lapinjärvi, Uusimaa region, southern Finland (60°39′6″ N, 26°8′17″ E, 57 m above sea level (a.s.l.))

Table 2: *Heterobasidion* species and strains used in this study

Fungal species	Strain/Genotype	Publications
H. parviporum	isolate 04009, homokaryotic	II
H. annosum	isolate 06068, heterokaryotic	III

3.2. Methods

Table 3: Methods used in this study

Methods	Publications
Growth conditions of *Heterobasidion annosum s.s.* and *H. parviporum*	II, III
Inoculation of saplings with *H. parviporum*	II

Methods	Publications
Growth conditions of Norway spruce (*Picea abies*) saplings	II
Growth conditions of Scots pine (*Pinus sylvestris*) trees	III
DNA isolation and sequencing	I, II
Genome-wide exon-capture analysis	II
Inoculation of trees with *Heterobasidion annosum*-colonized dowels	III
RNA isolation	III
cDNA synthesis	III
PCR	I, II, III
Gas chromatography and mass spectrometry	III
Tree genotyping	I, II
Field work	I, III, IV
Greenhouse work	II
Gene expression analysis	III
Terpenoid analysis	III
FT-IR analysis	IV
Statistical analysis	I, II, III, IV

4. RESULTS AND DISCUSSION

4.1. Mycobiota of symptomatic and asymptomatic Norway spruce trees naturally infected by *Heterobasidion* spp. (I)

4.1.1. The output of MiSeq sequencing and occurrence of *Heterobasidion*

Our sequencing effort resulted in 8,276,762 high-quality sequences. However, only 74 of 90 samples were selected for further analysis because of technical issues (smaller numbers of amplified sequences in the discarded samples). The number of high-quality sequences in the samples selected for the analysis ranged from 22,278 to 194,915, with an average of 103,390 ± 44,630 sequences. Two of the identified operational taxonomic units (OTUs), namely, Otu00011 and Otu00048, were assigned to the genus *Heterobasidion* and were tentatively classified as *H. annosum* and *H. parviporum*, respectively. In asymptomatic trees, Otu00048 was more abundant than Otu00011, whereas in symptomatic trees, Otu00048 had a lower abundance than Otu00011 **(I, Suppl. Table S1)**. The presence of *Heterobasidion* in asymptomatic trees is unlikely to be due to cross contamination during sample processing. It is possible that the trees were at the early stages of infection or the presence of dormant *Heterobasidion* spores, that earlier landed on the samples.

4.1.2. Richness, diversity and evenness of fungal communities of Norway spruce

The total number of OTUs identified in our survey was 4,375. The fungal communities in needle and root tissues had the highest richness in asymptomatic and symptomatic trees, respectively, whereas the bark tissue had the lowest richness in both symptomatic and asymptomatic trees **(I, Fig. 2A)**. In all sampled tissues, we found no significant differences in richness among asymptomatic and symptomatic trees. The needles had the highest community diversity, and the roots and lower stem had the lowest **(I, Fig. 2B)**. The bark and roots had the highest and lowest fungal species evenness, respectively **(I, Fig. 2C)**.

Furthermore, approximately 16.9% of the total OTUs were shared by all anatomic regions (needles, bark, upper stems, lower stems, and roots) of Norway spruce. The proportion of the OTUs unique to a certain anatomic region ranged from 1.6% (bark) to 13.3% (root) **(I, Fig. 2D)**. Principal coordinates analysis (PCoA) based on the relative abundance of fungal OTUs revealed differences in fungal community structure among anatomic regions of Norway spruce trees.

4.1.3. Impact of health status on the structure of fungal communities of Norway spruce

Our hypothesis was partially confirmed in that the fungal community structure significantly differed between asymptomatic and symptomatic Norway spruce trees. We found a significant difference among symptomatic and asymptomatic Norway spruce trees in only the upper stem and lower stem (P = 0.001 and P = 0.011, respectively) **(I, Suppl. Fig. S7)**. The fungal community structure was affected by *Heterobasidion* infection in the parts of the tree closest to the pathogen-colonized tissues, whereas there were no significant effects in more distant regions (**I, Supp Information Tables S2 and S3**).

Recently, Zampieri et al. (2017) found that *Heterobasidion* infection encourages mycorrhizal development in *Pinus pinea*. This result possibly indicates that *Heterobasidion* also has other effects on the fungal communities inhabiting the pathogen-infected trees. There are at least two plausible reasons for the lack of significant differences in fungal community structure in the root tissue between asymptomatic and symptomatic spruce trees in our study. First, mycorrhizal fungi constituted only a small fraction in our data set, and this low abundance is probably attributed to the fact that we collected suberized roots of Norway spruce trees instead of fine roots, where mycorrhizae occurs. Second, it is also possible that conifer tree species might react in different ways to *Heterobasidion* infection. The sampled standing spruce trees displayed no symptoms of infection. Symptoms were visible only after the trees were cut, and the decay symptoms were detected mostly in the heartwood. This

finding is in accordance with the fact that the development of *Heterobasidion* infection in Norway spruce is very slow, with the pathogen-infected trees showing few or even no symptoms. In Norway spruce, the changes in fungal community structure did not systematically show an association with the status of *Heterobasidion*-infected wood decay, at least at the early stages of infection.

4.1.4. OTUs with different relative abundances in asymptomatic and symptomatic trees

The saprotrophic and wood-degrading species were dominant in symptomatic trees, indicating that *Heterobasidion*-infected trees become more susceptible to coinfection with other wood-degrading fungi. The presence of these fungi was most likely affected by certain factors (e.g., wood structure, pH, moisture, and nutrient status), which invariably influence which species inhabit the host. Additionally, the developmental stage of the disease may also have strongly affected the tree microbiota.

This finding also demonstrated that 10 of 50 OTUs with the highest abundance differed in abundance in at least one of the sampled spruce tissues. The 10 OTUs listed in Table 1 of paper I were more abundant in asymptomatic trees (**I, Table 1 and Suppl. Table S1**). Three of these OTUs were *Hypogymnia tubulosa*, *Scoliciosporum umbrinum,* and *Phialocephala fortinii*. The most abundant fungus in symptomatic trees was *Talaromyces sp.*, which is a representative of a large genus of saprotrophic fungi commonly producing secondary metabolites. *T. flavus* also functions as a biological control agent for plant pathogens (Bahramian et al., 2016; Madi et al., 2007).

The OTUs found in asymptomatic trees comprised two lichen species (*H. tubulosa* and *S. umbrinum*) and a root endophyte (*P. fortinii*). The lichens had a higher abundance in asymptomatic trees and can produce secondary metabolites (Yılmaz et al., 2005). Ecological

functions of the *P. fortinii* species complex remain unknown, but its function is found widely in the roots of both ericaceous plants and conifers (Grünig et al., 2008). Members of *P. fortinii* can also produce secondary metabolites, mediating the plant inhibitory mechanism against pathogens (Tellenbach et al., 2013). Furthermore, Terhonen et al., (2016) demonstrated that Norway spruce seedlings can be protected *in vitro* from *H. parviporum* infection using a *Phialocephala* isolate.

4.2. Phenotyping and genome wide analysis of Norway spruce genes associated with necrotic lesion formation in response to *Heterobasidion parviporum* infection (II)

4.2.1. Phenotyping and heritability of Norway spruce to *H. parviporum* infection

We performed artificial fungal inoculation of saplings under greenhouse conditions to investigate the susceptibility of various clonal lines of Norway spruce to *Heterobasidion* infection. In our study, we examined differences in lesions developed in response to *H. parviporum* inoculation and in the mock treatment in both xylem and phloem tissues, and the treatments were significantly different ($P < 0.001$) (**II, Table 2**). Lesion size was significantly different among the examined clones, which concurs with the results of previous studies (Keriö et al., 2014; Skrøppa et al., 2015; Swedjemark et al., 2001; Swedjemark and Stenlid, 1996). Horizontal necrotic lesions in the phloem were significantly longer than those in the xylem ($P < 0.001$).

The present study also showed that Norway spruce saplings infected by *H. parviporum* with lower values of stem diameter, height, and volume (growth traits) had larger necrotic lesions. This finding is in accordance with previous studies (Karlsson et al., 2008), leading to the conclusion that the growth parameters had a strong positive correlation with resistance against *H. parviporum*. The largest lesions (the most susceptible) were observed in clonal lines V31094 and V31406.

The estimates of heritability for the lesion traits (e.g., phloem horizontal, phloem vertical, xylem horizontal, and xylem vertical necrosis) and growth traits (e.g., diameter, height, and volume) were moderate to high, with values of 0.18-0.80 **(II, Table 4).** Thus, the differences in environmental conditions had a relatively small effect on the expression of growth traits measured separately in the two treatments (lesion traits and growth traits). The estimates of heritability in the mock treatment were consistently larger than those in the

inoculation treatment, which is in line with the genetic coefficients of variations (GCVs) **(II, Table 4)**. These traits are therefore considered to differ genetically.

The combined data (two traits) revealed moderate heritability (**II, Table 5)**. The relative magnitude of the interaction showed that only phloem horizontal necrosis had high heritability, while the remaining lesion traits had low-to-moderate heritability and the growth traits had low heritability. This result deserves further examination in future studies, considering the small relative magnitude for all growth traits and almost all necrosis traits.

4.2.2. Identification of sequence variants correlated with necrotic lesion size

A genome-wide association study (GWAS) is an important tool used for the identification of genetic loci controlling economically valuable traits (Kump et al., 2011; Li et al., 2013; Tian et al., 2011; Wei et al., 2015) and has been applied to investigate the genetic regulation of various plant traits (Fahrenkrog et al., 2017). Resequencing of predicted exons from 64 clonal Norway spruce trees generated 34,761 gene models and 373,384 high-quality biallelic single nucleotide polymorphisms (SNPs). In addition, 34 genes associated with 36 SNPs were successfully identified using GWAS analysis, and the genes were significantly related to necrotic lesion size in response to *H. parviporum* inoculation **(II, Suppl. File S4)**. The 10 SNPs associated with control of the size of necrotic lesions of *H. parviporum*-infected Norway spruce are located within eight gene models **(II, Table 6)**. This finding of 10 SNPs located within eight genes that are significantly associated with larger necrotic lesions might be linked to the defense and susceptibility of Norway spruce to *Heterobasidion* infection. We assumed that the observed genes also play roles in the regulation of tree defense mechanisms in Norway spruce. Three plant defense-inducing genes are included in the spruce-genome gene models: MA_940838g0010, MA_4047g0010, and MA_3905g0010.

The first gene, MA_940838g0010, is similar to *Arabidopsis thaliana* ILITYHIA (ILA), previously described to be involved in the establishment of systemic acquired resistance (Monaghan and Li, 2010). This gene likely also functions in necrotic and programmed cell death in response to infection by phytopathogenic microbes (Faus et al., 2018). However, in conifer trees, including Norway spruce, the function of the protein has not yet been determined; hence, the functional relevance of the gene in the *Heterobasidion*-conifer pathosystem merits further study. The second gene (MA_4047g0010) encodes subtilase (subtilisin-like serine peptidase), which has multiple copies in many plant genomes. Some of its functions have been described, such as the control of growth and development (physiological function), plant responses to environmental factors (biotic and abiotic stressors), and the regulation of programmed cell death and senescence (Schaller et al., 2018). This gene had a higher expression level in symptomatic trees than in asymptomatic ones (p=0.06); however, we are unable to predict its specific roles, thus further experimental efforts are needed to determine the potential roles of this gene.

The third gene (MA_3905g0010) is homologous to a class of HD2-type histone deacetylases implicated in abscisic acid (ABA) and abiotic stress responses (Han et al., 2016; Luo et al., 2012; Sridha and Wu, 2006). It may play a key role in programmed cell death to regulate resistance or susceptibility to necrotrophic pathogens (Coll et al., 2011).

Additionally, this study revealed low-frequency alleles (<5%) in the evaluated genetic variants. Hence, a larger number of individual samples will be needed in genotyping programs to identify alleles associated with responses of *H. parviporum*-infected Norway spruce for use in breeding programs to screen and select tolerant trees.

4.3. Evaluation of potential genetic and chemical markers for tolerance to *Heterobasidion annosum* infection in Scots pine (III)

4.3.1. Development of necrotic lesions in Scots pine trees in response to *H. annosum* inoculation

Several studies involving artificial inoculations have been applied successfully to test tree resistance against pathogenic *Heterobasidion* in field experiments with different infection periods (Karlsson et al., 2008; Keriö et al., 2014; Swedjemark and Karlsson, 2004). In this study, the infection period was 5 months. The length of necrotic lesions in the phloem and xylem in both the vertical and horizontal dimensions and the sum of all lesion measurements were recorded. The necrotic lesion sizes varied and were normally distributed, indicating that each host genotype of individual Scots pine had a different degree of susceptibility to *H. annosum*. Furthermore, the length of necrotic lesions on the inoculated trees was significantly larger than that on the wounded controls ($P < 0.001$) (**III, Fig. 2** and **Suppl. Fig. S1**). This finding is in agreement with an earlier study (Danielsson et al., 2011) showing that lesions of Norway spruce infected by *H. annosum* were significantly longer after inoculation than those of wounded controls.

The sampled trees were subdivided into three groups: tolerant, intermediate, and susceptible trees, based on the sum of all necrosis. The necrotic lesions were significantly different both among the groups and between inoculation and wounding. Furthermore, the length of the necrotic lesions in the phloem (vertical dimension) that developed in response to *H. annosum* inoculation showed a significant positive correlation with the tree diameter (**III, Table 2 and Fig. 3**). This relationship suggests that trees of Scots pine with greater diameters are more sensitive to *H. annosum* infection. This is consistent with a previous study of Norway spruce (Swedjemark and Karlsson, 2004) that clearly showed diameter was positively correlated with both lesion length and fungal extension. However, this finding is in

contrast to an earlier study revealing that *H. parviporum* growth had a strong negative correlation with stem diameter in Norway spruce (Karlsson et al., 2008).

4.3.2. Analysis of gene expression profiles

Terpenoids are promising biochemical markers for the characterization of tree resistance against pests and/or diseases since terpenoids are among the most numerous compounds involved in chemical defense by conifer trees (Huber and Bohlmann, 2006). In the present study, we explored whether the terpenoid content and gene expression pattern in needles could be used as predictive markers for tree resistance against *H. annosum* infection.

Genes encoding α-pinene synthase and a putative (-)-alpha pinene synthase were significantly different among groups at $P = 0.012$ and $P = 0.027$, respectively **(III, Fig. 4(a) and Suppl. Table S2)**. Furthermore, Pearson's correlation between gene expression level and necrotic lesion size showed that genes encoding α-pinene synthase, a putative (-)-alpha pinene synthase, a predicted geranyl diphosphate synthase, and a homologue of metacaspase 5 (MC5) had a positive correlation with necrosis length **(III, Table 3** and **Fig. 5).**

In addition, the present study showed that the expression of the δ-3-carene synthase gene did not correlate with resistant or susceptible trees. This contrasts with the findings of other studies (Fäldt et al., 2003; Roach et al., 2014) in which δ-3-carene synthase expression was significantly correlated with resistant trees.

4.3.3. Terpene profiles of Scots pine needles

Both monoterpene and sesquiterpene concentrations varied in each necrotic lesion group. The highest concentrations among monoterpenes were found for *α*-pinene and *δ*-3-carene (mean >1,000 µg g^{-1}FW) (**III, Fig. 4b),** which is supported by earlier studies. The most abundant monoterpenes in Scots pine and whitebark pine were previously found to be δ-3-carene and α-pinene, respectively (Bullington et al., 2018; Vanhatalo et al., 2018). Among

sesquiterpenes, the compounds with the highest concentrations were *α*-muurolene and *β*-elemene (>250 µg g^{-1}FW) (**III, Fig. 4c)**. Another study (Muona et al., 1986) revealed that natural stands and trees of Scots pine contained 3-carene and other monoterpene compounds. Other authors also observed increased levels of 3-carene, myrcene, limonene and *β*-phellandrene in Scots pine trees (Baradat and Yasdani, 1988).

Additionally, none of the analyzed monoterpene and sesquiterpene compounds showed significant differences among the groups ($P > 0.05$) **(III, Suppl. Table S2)**. However, PCoA clearly differentiated between tolerant and susceptible trees (**III, Fig. 6).** Concentrations of β-caryophyllene and α-humulene had a negative correlation with the size of necrotic lesions, at $P = 0.05$ and $P = 0.068$, respectively (**III, Fig. 5e-h**, **Suppl. Fig. S3**, and **Suppl. Table S3)**. Other studies found the same compounds (β-caryophyllene and α-humulene) documented in the present study. β-caryophyllene was previously found to be the most abundant in maritime pine infected by maritime pine borer (*Dioryctria sylvestrella*) (Jactel et al., 1996). Similarly, other studies demonstrated that the sesquiterpenes β-caryophyllene and α-humulene released by Scots pine significantly increased when the common pine sawfly (*Diprion pini*) laid eggs on its needles (Köpke et al., 2010). The release of these terpenes attracts *Closterocerus ruforum*, an insect biocontrol agent, that parasitizes both eggs and larvae of *D. pini* (Köpke et al., 2010).

4.4. Fourier-transform infrared (FT-IR) spectroscopy discriminates between asymptomatic and symptomatic Norway spruce trees (IV)

This chemometric analysis is the first to use FT-IR spectroscopy for the analysis of Norway spruce naturally infected by *Heterobasidion* spp. Comparisons among different tissues (needle, phloem, and xylem) and between trees showing either the presence or absence of visible xylem decay symptoms (symptomatic and asymptomatic trees, respectively) were performed. For the tissue comparison, the mid-IR spectral regions (4,000–700 cm^{-1}) were collected from 54 biological samples in two technical replicates. Soft independent modeling of class analogy (SIMCA) analysis revealed that the spectral regions that can be used to discriminate between tissues are 1,066–912 cm^{-1}.

In addition, the interclass distance **(IV, Table 1)** and discrimination power were also used to optimize the SIMCA model. The greatest interclass distance (phloem versus xylem) and discrimination power were 11.14 and 968, respectively, and were obtained from the spectral region at 1,008 cm^{-1} assigned to carbohydrate (C-O) (Conrad et al., 2014; Martin et al., 2008; Pandey and Theagarajan, 1997). Since the acquired absorbances in certain spectral bands were different among the needle, phloem, and xylem samples, the chemical compositions of these tissues differed. This result agrees with a previous study (Antonova and Stasova, 2006) that showed phloem and xylem of Scots pine had significantly different compositions of amino acids, carbohydrates, organic acids, and phenol compounds. Another study revealed that resin and polyphenolics differed significantly among the needle, phloem, and xylem tissues in *Pinus radiata* juveniles (Moreira et al., 2012).

A comparison of xylem, phloem, and needles of symptomatic and asymptomatic trees was also performed. As indicated earlier, asymptomatic refers to trees without visible decay symptoms in their sapwood and heartwood at the stump level, whereas symptomatic refers to trees with well-defined decay symptoms. In xylem tissues, SIMCA analysis distinguished

between asymptomatic and symptomatic trees based on spectral bands at 1,657–994 cm^{-1} (**IV, Fig. 4**), and the interclass distance was 2.69. These spectral bands were primarily associated with carbohydrate (C-O), carbonyl (C=O), and benzene (C=C) groups stretching vibrations (Conrad et al., 2014).

Furthermore, the highest discriminating power was 134 and 104 in the spectral regions at 1,081 cm^{-1} (assigned to cellulose and hemicellulose) and 1,432 cm^{-1} (assigned to lignin and carbohydrates), respectively (**IV, Fig. 4B**) (Pandey and Theagarajan, 1997). This finding is in accordance with a previous study (Villari et al., 2018) revealing that FT-IR spectroscopy using SIMCA analysis (spectral regions from 748 to 798 cm^{-1} and from 879 to 947 cm^{-1}) can differentiate between resistant and susceptible trees of European ash inflicted with ash dieback disease.

FT-IR spectroscopy can also discriminate among symptomatic, asymptomatic, and healthy leaves of adult trees of sweet orange infected by the two pathogens, *Xylella fastidiosa* and *Candidatus* Liberibacter spp., for which obtained spectral bands were at 1,175–900 cm^{-1}, corresponding to the absorption of starch (Cardinali et al., 2012). In addition, a previous study of Scots pine sapwood decayed by *Coniophora puteana* showed spectral regions in the FT-IR at 1,596, 1,511, 1,268 and 1,220 cm^{-1} that significantly increased in association with lignin alteration (Pandey and Pitman, 2003).

Needle extracts of Norway spruce can be used to differentiate between asymptomatic and symptomatic trees using spectral regions from 1,104–994 cm^{-1} (**IV, Fig. 5**), which are associated primarily with carbohydrate (C-O) group stretching vibrations (Conrad et al., 2014). The highest discriminating power (95) and interclass distance (2.75) were found in the spectral band at 1,016 cm^{-1}, corresponding to the stretching of starch (C-O) (Martin et al., 2008) and cellulose and hemicellulose (Pandey and Theagarajan, 1997). In addition,

information obtained from FT-IR analysis can be used to follow changes in the physical and chemical composition of infected trees (Fackler and Schwanninger, 2012).

In the present study, spectra recorded from phloem extracts did not discriminate between asymptomatic and symptomatic trees in SIMCA analysis. Nevertheless, the condensed tannin concentration (mg g^{-1} DW) in phloem extracts can be predicted using a partial least squares regression (PLSR) approach and two transformation models (second derivative and normalized) from spectral bands at 1,680–1,279 cm^{-1} (**IV, Fig. 6**). This finding is in agreement with two earlier studies by Schwanninger et al. (2011) and Zhou et al. (2011), that applied PLSR to predict the total Norway spruce lignin content as well as predict the lignin and energy contents in hybrid poplar (*Populus trichocarpa* × *P. deltoides*). The predicted tannins observed in the present study are defense-related chemical compounds (War et al., 2012) that act as a general antimicrobial defense in microbial interactions (Constabel et al., 2014; He et al., 2008).

Finally, we identified spectral regions of the xylem (1,657–994 cm^{-1}) and needles (1,104–994 cm^{-1}) that can be used to discriminate between asymptomatic and symptomatic trees and spectral regions of the phloem (1,680–1,279 cm^{-1}) that predicted the condensed tannin concentration. The different spectral regions reflect different chemical compositions and contents in Norway spruce trees, which are perhaps attributable to infection by *Heterobasidion* spp.

5. SUMMARY AND FUTURE PERSPECTIVES

In conclusion, in paper **I**, we observed the effect of a Norway spruce pathogen (*H. parviporum*) on the composition of its mycobiota. This study proved that the progression of the root and stem rot disease significantly changed the structure of the resident microbial communities. However, the root and stem rot disease did not affect the mycobiota of more distant tree tissues, as the effect was rather localized. Additionally, each tree tissue was found to have a unique mycobiota. Symptomatic trees also appeared more likely to be colonized by other wood-degrading fungi.

In papers **II** and **III**, we phenotyped clonal Norway spruce saplings and mature Scots pine trees showing different levels of susceptibility to *H. parviporum* and *H. annosum*, respectively. Gene expression and Genome-wide association analysis using sequence capture revealed several genes, which are considered promising marker candidates for future screening programs. The role of defense reactions of spruce warrants exploration in further studies (paper **II**).

Our study identified genes predicted to encode α-pinene synthase (two genes), geranyl diphosphate synthase (GPPS), metacaspase 5 (MC5), and the two terpenoid compounds β-caryophyllene and α-humulene associated with necrotic lesion formation. The investigated genes and terpenoid compounds can be used as potential markers of tolerance/susceptibility of Scots pine to *H. annosum* infection. The unknown roles of these genes and compounds associated with pine defense merit further study, which will support tree selection and breeding programs (paper **III**).

The chemometric study is the first example of the application of the FT-IR technique to analyze Norway spruce trees naturally infected by *Heterobasidion* spp. Our results indicate the potential applicability of FT-IR in the determination of the health status of spruce trees (paper **IV**).

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